The functions of cytokines and their uses in toxicology

JOHN R. FOSTER
AstraZeneca plc, Macclesfield, UK

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Summary. Cytokines are critical controllers of cell, and hence tissue, growth, migration, development and differentiation. The family includes the inflammatory cytokines such as the interleukins and interferons, growth factors such as epidermal and hepatocyte growth factor and chemokines such as the macrophage inflammatory proteins, MIP-1α and MIP-1β. They do not include the peptide and steroid hormones of the endocrine system. Cytokines have important roles in chemically induced tissue damage repair, in cancer development and progression, in the control of cell replication and apoptosis, and in the modulation of immune reactions such as sensitization. They have the potential for being sensitive markers of chemically induced perturbations in function but from a toxicological point of view, the detection of cytokine changes in the whole animal is limited by the fact that they are locally released, with plasma measures being generally unreliable or irrelevant, and they have short half lives which require precise timing to detect. Even where methodology is adequate the interpretation of the downstream effects of high, local concentrations of a particular cytokine is problematic because of their interdependence and the pleiotropism of their action. A range of techniques exist for their measurement including those dependent upon antibodies specific for the respective cytokines, but with the introduction of genomic and proteomic technology, a more complete study of cytokine changes occurring under the influence of chemical toxicity should be possible. Their further study, as markers of chemical toxicity, will undoubtedly lead to a greater understanding of how synthetic molecules perturb normal cell biology and if, and how, this can be avoided by more intuitive molecular design in the future.

Keywords: Cytokines, growth factors, apoptosis, signal transduction, cell proliferation, cancer, chemical allergy, inflammation, assays

Correspondence: Dr John R Foster, Safety Assessment, Astra Zeneca plc, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK. Tel.: 44(0) 1625 582828; Fax: 44(0) 1625 583074; E-mail: John-Foster@astrazeneca.com

Introduction

Cytokines are a broad group of signalling proteins that are produced transiently, after cellular activation, and act as humoral regulators which modulate the functions

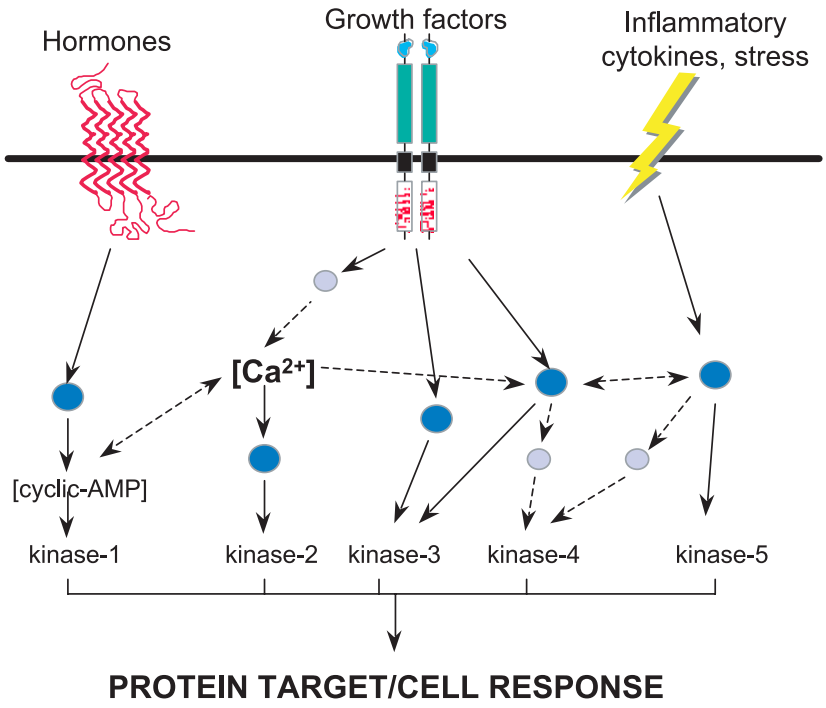

Figure 1. Model of how the phenomenon of 'cross talk' may be envisaged as taking place. The dotted arrows indicate routes of cross-talk. The increases observed in Ca^{2+} and cyclic-AMP, together with many other molecules, are common to several different ligand–receptor interactions, as is the phosphorylation of the individual kinases. The circles represent putative intermediate messengers which may or may not be common to any particular pathway.

of individual cells, and regulate processes taking place under normal, developmental and pathological conditions (Dinarello *et al*. 1990; Meager 1998). They are able to act locally, as autocrine, juxtacrine or paracrine response modifiers, and their action is initiated via specific receptors expressed primarily on the cell membranes of their target cells (Miyajima *et al*. 1992). Unlike hormones, cytokines are produced by cells which are not organized in special glands and which act systemically to affect biological phenomena such as inflammation, wound healing, organogenesis and oncogenesis.

The word *cytokine* is derived from the Greek *kytos* meaning 'hollow' or 'vessel' and *kinein* meaning 'to move' and was originally used to separate a group of immuno-regulatory proteins, such as interleukins, from other chemicals, known as *growth factors*, that modulated the proliferation and bioactivation of nonimmune cells. As more has become known about these proteins however, it has become increasingly obvious that the distinction of these two terms is artefactual and many of the classical immuno-modulatory cytokines are able to effect proliferation and differentiation in both immune and nonimmune cells, while many of the so-called typical growth factors are able to regulate immune competent cells both directly and indirectly.

A third group of soluble chemo-attractant cytokines have been termed *chemokines* and one of the first to be described was interleukin-8 (IL-8). The chemokine group consists of a large number of proteins, all with their respective receptors, and includes molecules such as RANTES (regulated on activation, normal T expressed and secreted), the monocyte chemotractant proteins (MCPs), and lymphotactin (Meager 1998). They are all concerned with the recruitment and activation of immuno-competent and inflammatory cells into sites of cell damage.

With some notable exceptions, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), most cytokines are not stored in cells and their expression is strictly regulated, being produced only by activated cells in response to an induction signal. Expression is normally transient although constitutive expression, for example of epidermal growth factor (EGF), has also been demonstrated in some cells (Wong & Wright 1999). Almost all cytokines elicit multiple biological activities in multiple cell types and different cytokines have been shown to have overlapping activity with a single cell type seemingly able to show identical responses to multiple cytokines (Sun *et al*. 1999). The term 'cross-talk' (see Figure 1) has been coined to describe such phenomena (Paris *et al*. 1988). A consequence of functional overlap is that one factor may replace another factor or compensate for the lack of particular factor, and since most cytokines have

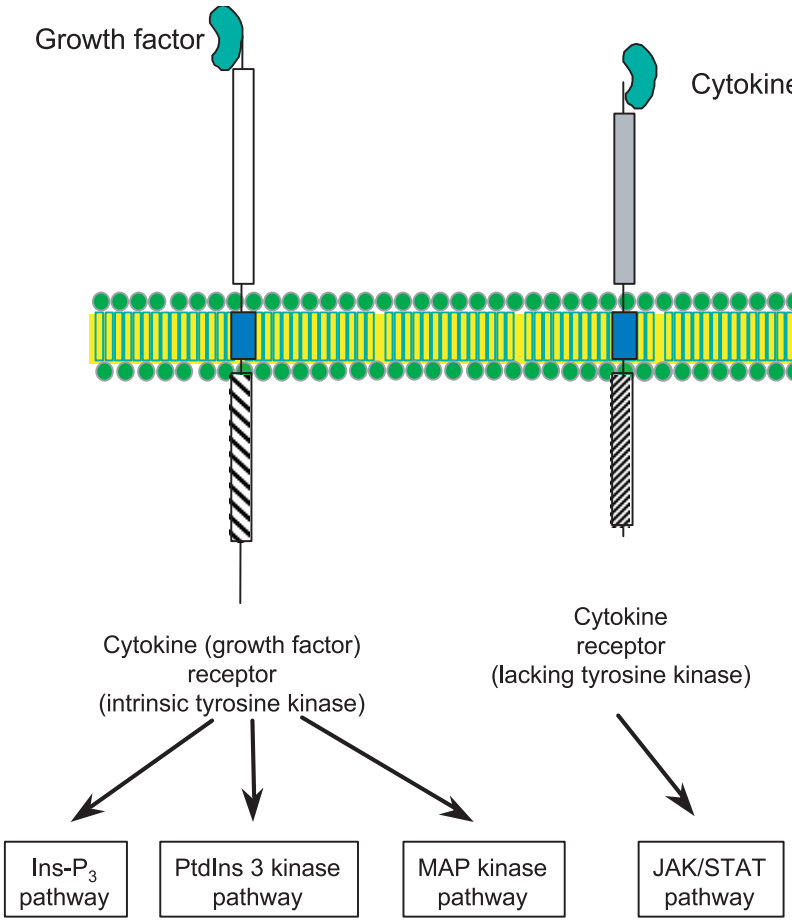

Figure 2. Model of signal transduction from cytokine cell surface receptors with either intrinsic protein kinase activity (classical growth factors), or lacking protein kinase activity (inflammatory cytokines). InsP3 = inositol 1,4,5-trisphosphate; PtdIns 3-kinase = phosphatidyl inositol 3-kinase; MAP kinase = mitogen-activated protein kinase; JAK-STAT = Janus kinases and signal transducers and activators of transcription.

ubiquitous activities their significance as regulators of normal physiology can be difficult to assess, particularly *in vivo*.

Most cytokines show stimulatory or inhibitory activities and may synergise or antagonize the actions of other cytokines and hormones (Matsumoto & Kanmatsuse 2000). An important feature of their action is that a single cytokine may induce one type of reaction under one circumstance and elicit entirely the opposite reaction under a different set of circumstances (Sun *et al*. 2000). This has particular relevance in their therapeutic use. The growth state of the recipient cells, the environment in terms of neighbouring cells, the cytokine concentration and the combinations of other cytokines present can all influence the type, duration and extent of reaction shown (Hasbold *et al*. 1999).

The biological activities of cytokines are mediated by specific membrane receptors which can be expressed on virtually all cell types. The mechanism by which receptor occupation by cytokines results in the generation of a signal through the receptor is not completely understood. It is likely that on binding to the extracellular portion of the receptor the cytokine induces a conformational change, or oligomerization of multiple receptors, resulting in an activation event in the intracellular domain itself or in receptor-associated elements such that signal transduction to elicit subsequent intracellular events occurs (Figure 2). Small antagonist molecules that bind to cytokine receptors do not activate subsequent events probably because they do not deform the extra-cellular receptor sufficiently to excite subsequent events (Meager 1998). Two major signal transduction pathways are principally involved in cytokine activation and both result in activation of protein kinases that catalyse the transfer of high energy phosphate onto protein substrates (phosphorylation) which subsequently act for secondary signal transduction and amplification. The first pathway, used by mitogenic cytokines such as EGF, contains tyrosine kinases as the primary signal transducers which may or may not be directly linked to the intracellular receptor depending upon the cytokine involved. The second

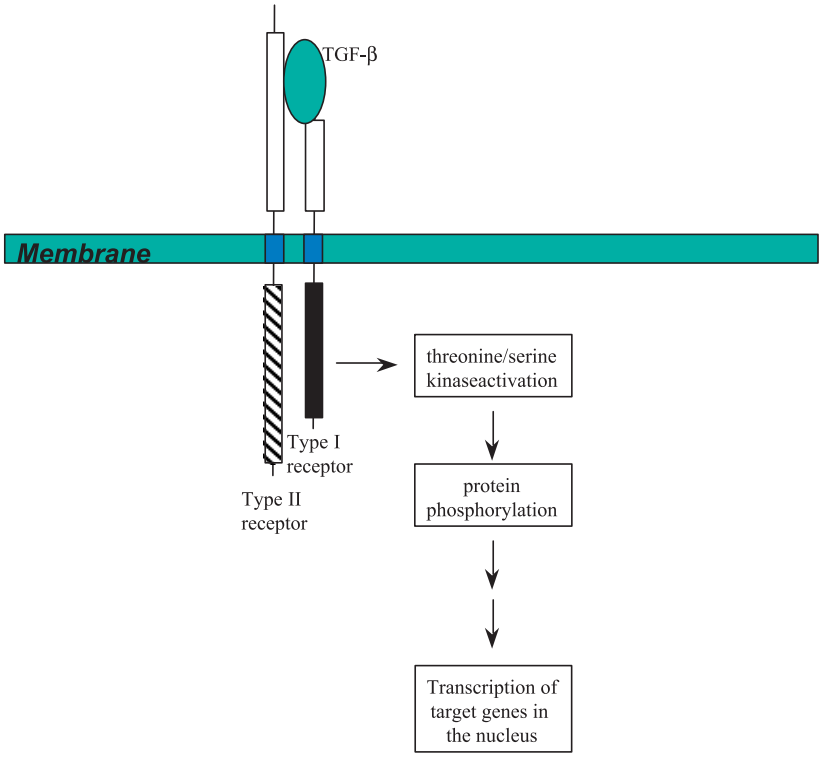

Figure 3. Signal transduction following binding of TGF-β to its receptor on the cell membrane. Binding results in phosphorylation of threonine/serine kinases and subsequent activation of target genes in the nucleus of the cell.

pathway involves activation of phospholipases, which in turn produce small mediators that activate serine-threonine kinases and raise intracellular calcium, and is used by TGF-β (Figure 3). Both of the major pathways also involve downstream effector molecules such as GDP-binding proteins, calcium-binding proteins, protein phosphatases and the products of proto-oncogenes. Even though the receptors may differ, those to different hormones and cytokines can, on binding their respective ligand, share common signal transduction pathways. It is probable that this phenomenon may explain the functional redundancy, and the pleiotropic response that certain cytokines elicit (Figure 1).

Although a variety of cytokines are known to share some biological effects the observations that single cells *in vitro* show different patterns of gene expression in response to different cytokines can be taken as evidence for the existence of cytokine-specific, receptor signal transduction pathways. Shared and separate transcriptional activators, that transduce a signal from a cytokine receptor to a transcription regulatory element of DNA, are involved in these processes, e.g. STAT proteins (Kotenco and Pestka 2000).

The regulation of cytokine function is not well understood but it is frequently possible to observe a hierarchical order of cytokine actions with some early cytokines preactivating cells so that they can then respond to later acting cytokines (McKay *et al*. 2000). A close examination of the physiological and pathological effects of regulated and deregulated expression of cytokines has shown that these mediators are involved in virtually all general systemic reactions of an organism including the regulation of immune responses, inflammation, haematopoiesis and wound healing. Cytokines, such as hepatocyte growth factor (HGF), are important mediators in embryogenesis and organ development and mice genetically altered such that they don't express HGF ('knockout' mice) die *in utero* with a grossly underdeveloped liver (Schmidt *et al*. 1995). This example illustrates that the way that certain cytokines act in mature animals may differ significantly from that observed postnatally (Dickson & Salomon 1998) since HGF in the adult is known to have multiple effects on organs including the liver and kidney in regulating cell numbers (Hoffman *et al*. 1994; Balkovetz & Lipschutz 1999).

Cytokines as markers of cytotoxicity

In many acute and chronic inflammatory diseases in humans, such as hepatitis, rheumatoid arthritis and meningitis, plasma measurements have shown large increases in a broad range of cytokines (Arvidson *et al*. 1994; Vindenes *et al*. 1998), and monitoring changes in

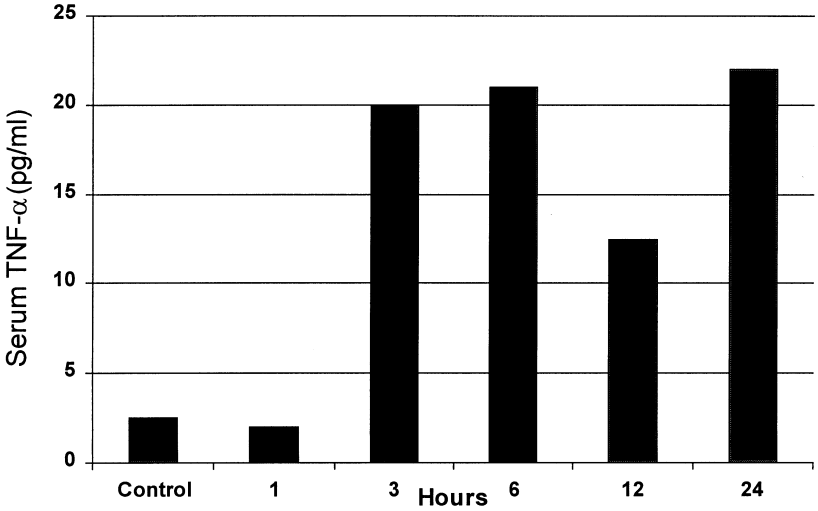

Figure 4. A time course of changes in the plasma concentration of TNF-α in mice administered a hepato-necrotic dose level of paracetamol. Necrosis was initially seen at 3 h and remained for the duration of the study. (adapted from Simpson *et al.* 2000).

these levels in, for example, synovial fluid in the case of arthritis (Feldmann *et al.* 1996), can allow an indirect measure of the progression of the disease in response to therapy or in running its natural course (Zissel *et al.* 1999; Simpson *et al.* 2000). In terms of their use in monitoring toxicity under certain conditions, and depending on the correct choice of cytokine to study, their measurement may be a sensitive indicator of the interaction of the drug/chemical with tissues and organs (Anscher *et al.* 1998; Robinet *et al.* 1995). An example of the application of this technique is given by Simpson *et al.* (2000) where a cytotoxic dose of paracetamol to mice has been shown to be accompanied by increases in plasma TNF-α which coincide with the appearance of centrilobular necrosis in the liver (Figure 4).

Dose–response relationships were clearly shown for changes in the induction of the mRNA for both IL-1β and TNF-α in the livers of mice given 2,3,7,8-tetrachlorodibenzo-p-dioxin (Fan *et al.* 1997) and the study also provided no effect levels for the cytokine responses to TCDD (Figure 5). The majority of experiments where dose–response relationships between chemical and cytokine response in cells involve *in vitro* systems and it is highly probable that the tighter controls possible in such systems allows a more precise examination of changes in cytokine concentration in the culture medium, and in the cell expression of receptors, than equivalent measures do in the plasma of humans or other animals. A possible explanation for any discrepancies between *in vitro* and *in vivo* assays may lie in the use of plasma measures when in the majority of cases the cytokine response is occurring locally in the affected tissue or organ, where measures of cytokine changes, if possible, would be more specific and almost

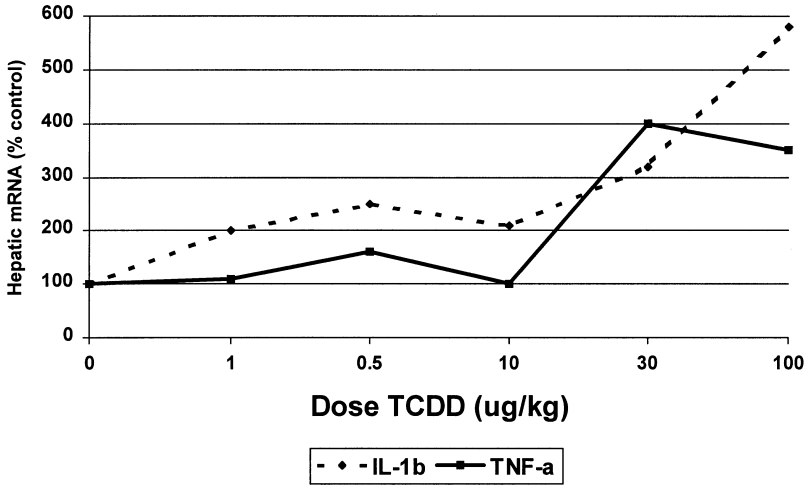

Figure 5. The dose–response relationship between TCDD and induction of the mRNAs of IL-1β and TNF-α in rat liver (adapted from Fan *et al.* 1997).

certainly more quantitatively related to the extent of damage occurring in the target tissue. When immunohistochemistry techniques were used to study phenobarbital-induced changes in the spatial distribution of cytokines such as TGF-β1, TGF-α, EGF-receptor and the mannose-6-phosphate receptor, their expression was altered in a way consistent with the known regional effect that this compound has on other bio-markers in rodent liver (Orton *et al*. 1996).

Cytokines and cancer

Cancer is a disease induced by a failure of cells to differentiate, coupled with a breakdown in the appropriate control of cells entering the cell cycle. Early data from transgenic mice coexpressing the cytokine, TGF-α, with either the oncogene, c-myc, or the simian form of the c-H-ras gene, showed that the additional over-expression of TGF-α was associated with an increased, and earlier, appearance of hepatic and pancreatic cancer when compared to littermates transgenic in either of the oncogenes only (Sandgren *et al*. 1989, 1993).

Cytokines are known to be important negative and positive regulators of cell replication, differentiation, migration, cell survival, cell death and cell transformation (de Kretser *et al*. 1998; Kakeya *et al*. 2000). There are also well recognized relationships between cytokines and oncogene expression and many of the receptors for the growth factor group of cytokines are protein products of oncogene expression. Over-expression of certain cytokine receptors, such as erbB2 (neu) in transgenic mice has been shown to be associated with tumour formation (Guy *et al*. 1992), and expression of *c-myc* oncogene in combination with over-expression of TGF-α results in a more severe cell dysplasia and a shortening in the time to tumour interval in the liver of transgenic mice than was seen with either transgene expressed separately (Santoni-Rugiu *et al*. 1996).

In normal tissues cytokines control the entry of cells into the cell cycle and hence the cell replicative process (Hirano *et al*. 2000; Kalvakolanu 2000; Kleeman *et al*. 2000). That cancer proceeds by a number of different stages is a broadly accepted principal and at virtually every stage in the development of cancer, cell replication is a critical determinant of the speed at which growth independence and the evolution of metastatic differentiation characters appear (Foster 1997; Cohen 1998). Indeed a loss of control of the balance between cell replication and cell death is thought to be one of the features that predispose the cells within a tissue to accumulating mutations and to develop growth independence and become neoplastic (Finlay 1993; Foster 1997; 2000).

Changes in the expression of receptors to the growth factor group of cytokines in cancer development have been widely reported (McClain *et al*. 1995; Dickson & Salomon 1998) and involve tumours of the reproductive tract, mammary gland and prostate of humans, as well

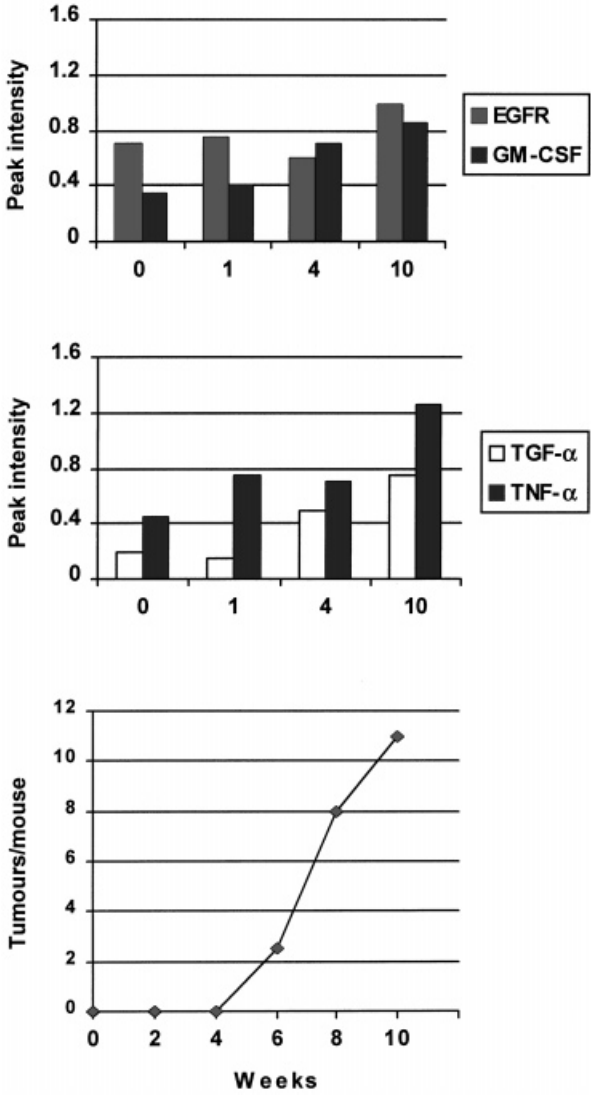

Figure 6. The correlation between the induction of skin papillomas in TgAC mice by arsenic and plasma levels of various cytokines (adapted from Bruccoleri *et al*. 1997). EGFR = epidermal growth factor receptor; GM-CSF = granulocyte macrophage colony stimulating factor; TGF-α = transforming growth factor alpha; TNF-α = tumour necrosis factor alpha.

as spontaneous tumours of laboratory animals and those induced by chemical carcinogen treatment (Yuspa *et al*. 1995; Digiovanni *et al*. 1994; Romach *et al*. 1997). Although up-regulation of receptors is seen in developing cancer cells, in the phenobarbital-induced rodent liver model for cancer promotion the hepatocytes become refractive to the mitogenicity of the drug by virtue of a down-regulation of receptors to EGF and a loss of the ability of protein kinase C to be translocated to the plasma membrane (Eckl *et al*. 1988; Meyer & Jirtle 1989; Brockenbrough *et al*. 1991; Orton *et al*. 1996). During the subsequent development of hyperplastic hepatocellular foci, over-expression of TGF-α in the foci has been reported in livers initiated with dimethylnitrosamine and subsequently given phenobarbital (Kaufmann *et al*. 1992). This does not however, appear to be a universal feature of hyperplastic liver foci since examination of morphologically similar foci from rats given diethylnitrosamine followed by the peroxisome proliferator, WY-14,643, did not over-express TGF-α (Miller *et al*. 1995). These results advise caution in over-interpreting the significance of such changes in limited circumstances.

In terms of the use of cytokines as markers of tumour development some attempt has been made to correlate changes in cytokine concentrations using the transgenic TgAC mouse strain where the induction of skin papillomas, by a combination of phorbol ester treatment and arsenic, was accompanied by increased concentrations of TNF-α and TGF-α during the development of the tumours (Figure 6) whereas no changes were seen in the expression of the receptor to epidermal growth factor (EGFR) and GM-CSF (Germolec *et al*. 1998).

The relatively limited amount of studies where cytokine expression has been followed during the development of cancer has indicated that the responses do not involve all cytokines, that those involved are not merely those associated with cycling cells, and that some functional relationship between the stage in tumour development and the particular cytokine undergoing altered expression can be determined (Frauman & Moses 1990; Michiel & Oppenheim 1992). The major limitation to *in vivo* studies appears to be the reliance on surrogate plasma measures of cytokines rather than those targeted to the site of tumour development. Future *in vivo* studies should provide more physiologically relevant data on cytokines by targeting the investigations to changes occurring within the developing tumour tissue since up to the present *in vitro* studies albeit on short-term cytotoxic phenomena and not cancer, have shown the most promise in providing interpretable data where cytokines are concerned.

Cytokines and cell proliferation

A large number of cytokines, including many in the growth factor category, are directly mitogenic for their target cells. They do so by activating common or convergent signalling pathways that induce similar effector molecules. The proto-oncogenes c-fos and c-myc are commonly involved in the transcriptional control of mitogenesis and they have been shown to be involved in the PDGF-induced mitogenic response, and probably so for most, if not all, of the other growth factor cytokines (Barone & Courtneidge 1995).

Given appropriate dose levels most drugs and chemicals prove to be toxic, the majority targeting to specific organs and tissues (Lock 1995; Pelkonen & Raunio 1997). Cytotoxicity, resulting in necrosis, is one manifestation of an adverse biological effect and chemicals, such as carbon tetrachloride and paracetamol, have been shown to cause necrosis in the liver when appropriate dose levels are used (Gomez *et al*. 1975; Lee *et al*. 1998). Any situation where cell damage occurs as a result of chemical exposure will give rise to inflammation, cell migration and aggregation to the site of the damage and the controlling molecules for these reactions are cytokines. Hence monitoring cytokine concentrations *at the site of damage* has the potential for monitoring the extent of cytotoxicity, and possibly relating, in a more specific way, the type of cytotoxicity occurring.

Other chemical classes, or lower dose levels of frankly cytotoxic chemicals, induce organ specific growth without causing cell death, and in the liver at least these are often, although not always, associated with the induction of drug metabolizing enzymes of various sorts (Lake *et al*. 1998). Drugs such as sodium phenobarbitone, and the peroxisome proliferating class of chemicals, such as nafenopin and Wy14,643 (Price *et al*. 1992; Coni *et al*. 1993; Rumsby *et al*. 1994; Miller *et al*. 1996), induce this type of response in the liver of rodents. In all of these cases the liver growth is accompanied by DNA synthesis, in both the hepatic parenchymal cells and in the vascular and Kupffer cell components of the liver, and is not associated with cell damage as a primary event (Roberts *et al*. 1995; Barrass *et al*. 1993).

In cases of induced cell replication, whether from chemicals causing cell death or from mitogenic agents, where proliferation is not preceded by cell death, the role of cytokines at multiple stages in the initiation, control

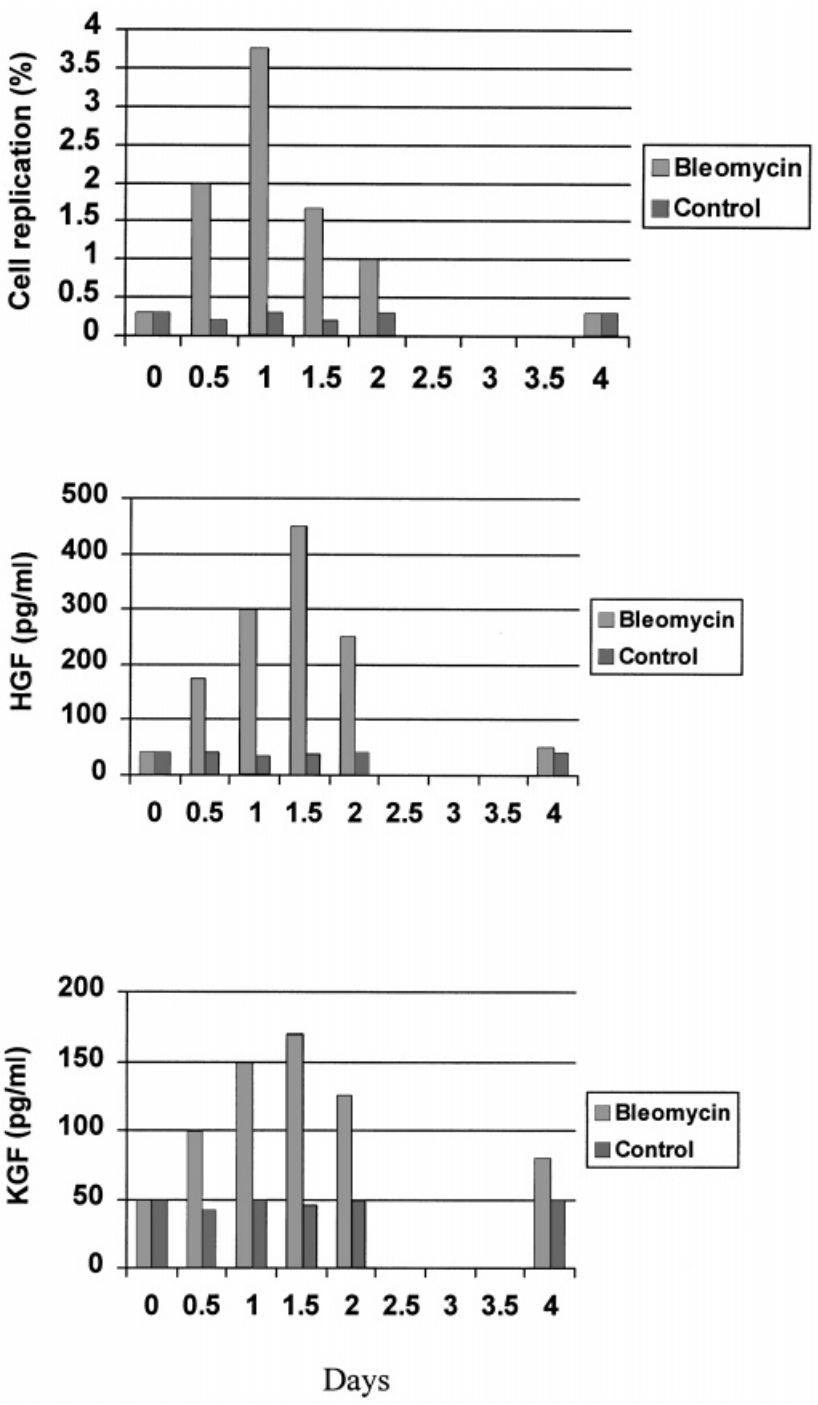

Figure 7. The relationship between the induction of cell proliferation in rat lung, following administration of bleomycin, and HGF and KGF cytokine concentrations in the plasma. MI = mitotic index. (adapted from Adamson & Bakowska 1999).

and cessation of the response will be critical in ensuring that the induced cell replication is correctly regulated. The early cell replicative response that occurs in the liver in response to sodium phenobarbital has been shown to be associated with increased expression of TGF-α, EGF and both HGF and its receptor with a concomitant decrease in the expression of TGF-β1 and the receptor to mannose-6-phosphate (Orton *et al*. 1996; Roberts & Kimber 1999). However continued administration of phenobarbital has been shown to result in the increased expression of other cytokines, including TGF-β, in the liver of rats at a stage when the cell proliferation

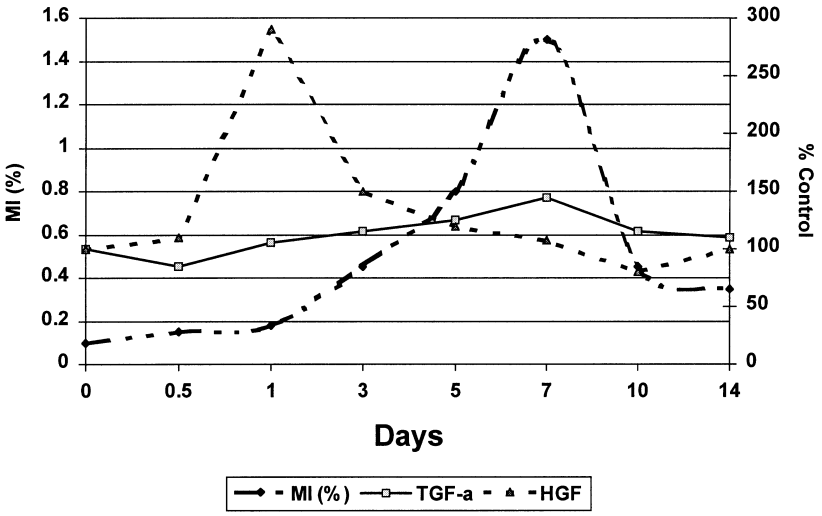

Figure 8. A time course for the induction of TGF-α and HGF in the liver of rats given sodium phenobarbitone. A comparison with the induction of mitosis. MI = mitotic index (adapted from Tomiya *et al*. 1998).

response had largely disappeared (Abanobi *et al*. 1982; Jirtle *et al*. 1991; Andersen *et al*. 1995). These cytokine changes are thought to be pivotal in the management of the cell replicative process that occurs in response to this type of chemical.

The cytokines, IL-1a and IL-1b, have been shown to have critical roles in terminating the cell replicative response that occurs in the liver, following partial hepatectomy in rats, such that the liver, on attaining its correct size, ceases further cell replication rather than continuing to grow (Boulton *et al*. 1997). Of equal importance in these studies was the observation that the source of the cytokines was not the hepatocytes themselves but the nonparenchymal cells, i.e. the Kupffer and endothelial cell components of the liver. Fausto *et al*. (1991) similarly showed that the origin of TGF-β production in the liver is also the nonparenchymal cell population.

Exposure of rodents to the hepato-necrotic agent, carbon tetrachloride, is associated with increases in the hepatic expression of TGFα and TNF-α (Heller & Kronke 1994), but it remains unclear exactly where, in the process of degeneration and regeneration, the cytokines act (Bruccoleri *et al*. 1997; Yamada & Fausto 1998). Logic dictates that these pro-inflammatory cytokines will function in both processes of cell damage/inflammation and in the subsequent regulation of cell proliferation that occurs as the reparative process proceeds. Indeed studies using mice, where one of the receptors for TNF-α, TNFR1, was deleted by gene knockout technology, have shown that the mice were subsequently unable to undertake liver regeneration following either partial hepatectomy or dosing with hepatotoxic concentrations of carbon tetrachloride (Yamada *et al*. 1997; Yamada & Fausto 1998). Antibodies to TNF-α have further been shown to inhibit the DNA synthesis induced by the simultaneous administration of the peroxisome proliferating chemical, Wy 14,643, to mice (Bojes *et al*. 1997). These studies have suggested that TNF-α is essential for the correct control of the proliferative response in the rodent liver following either cell loss or enzyme induction but exactly where in the process it acts, and what other cytokines are involved, remains unclear.

Studies in rats given the pneumotoxin, bleomycin (Figure 7) have shown a good correlation between the onset and peaks in cell replication, as measured by incorporation of BrdU, and increases in the plasma concentrations of both hepatocyte growth factor (HGF) or keratinocyte growth factor (KGF) (Adamson & Bakowska 1999).

Sodium phenobarbitone administration has also been shown to induce HGF levels in the liver which correspond with increases in the mitotic index measured at the same time (Figure 8). However concentrations of TGF-α did not change following exposure to sodium phenobarbitone even though partial hepatectomy, a surgical resection of the liver where very high levels of cell replication are induced, caused increases in both TGF-α and HGF (Figure 9) which corresponded with peak measures in the mitotic index in the liver (Tomiya *et al*. 1998).

These experiments only serve to illustrate that the same end point, DNA synthesis and cell replication, can be achieved under very different cytokine controls dependent upon the stimulus provoking the cell replicative response. This also underlines the need to monitor a range of cytokines when studying the replicative response rather than focus on any single protein since it can be difficult to predict exactly which will be involved in any one response.

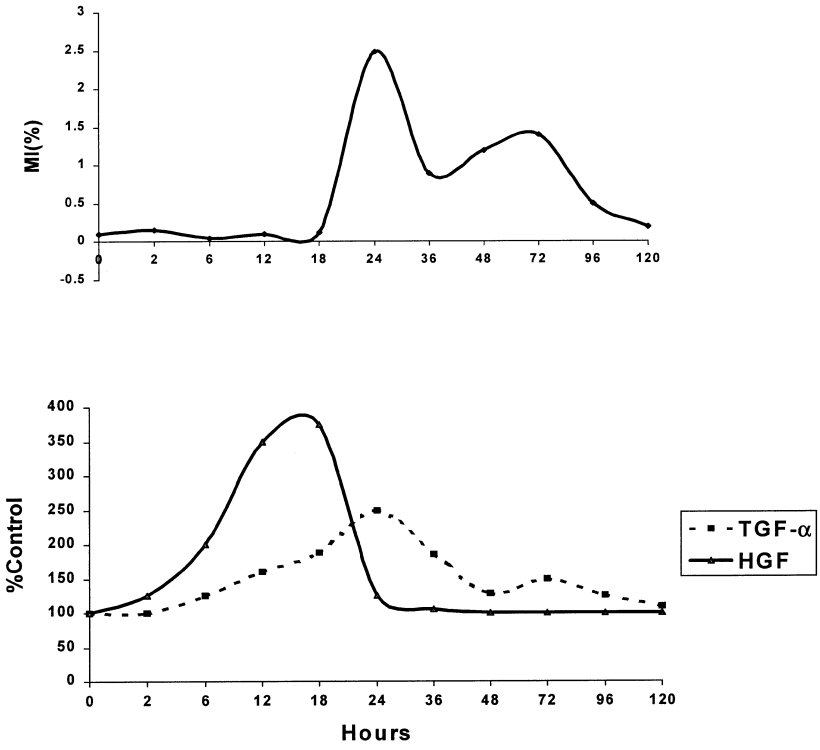

Figure 9. A time course for the induction of TGF-α and HGF in the liver of rats following 2/3 partial hepatectomy. A comparison with the induction of mitosis. (adapted from Tomiya *et al*. 1998).

Cytokines and apoptosis

It is well recognized that apoptosis can be induced *in vitro* by removing serum from the culture medium and it was soon discovered that this was due to depriving the growing cells of cytokines (Baffy *et al*. 1993; Magnelli *et al*. 1993; Broome *et al*. 1995). Figure 10 is a schematic of how cytokines may interact with receptors to provoke the inhibition, or initiation of apoptosis. Hence cytokines such as HGF, IGF-1, TGF-α and IL-2 were associated with a promotion of cell replication, and hence cell survival, *in vitro* while suppressing apoptosis. In contrast other cytokines such as TGF-β, on addition to cells in culture, induced increased apoptotic rates and were known as positive regulators of apoptosis in such systems.

The regulation of apoptosis has been considered an important component of chemically induced organ growth (Foster 2000) but has probably received even more attention in its postulated role in the development of neoplasia (Martin & Green 1995; Schulte-Hermann *et al*. 1998; Reed 1999; Engelmann & Bauer 2000; Kauffman & Gores 2000). TGF-β, when added to cultures of hepatocytes is a positive initiator of apoptosis (Strange & Roberts 1996; James *et al*. 1998) while chemicals which cause liver growth, such as sodium phenobarbitone and nafenopin, have been shown, both *in vivo* and *in vitro*, to decrease the apoptotic rate in rodent liver while reducing the expression levels of TGF-β (Christensen *et al*. 1999). The inhibition of apoptosis in the FTO-2B cell line *in vitro* by a variety of chemicals, including clofibrate, dieldrin and DDT, has also been shown to be accompanied by a decrease in the concentration of TGF-β secreted by these cells (Buchmann *et al*. 1999). In detailed studies on the mechanism of how the peroxisome proliferator group of chemicals inhibit apoptosis, Hasmall *et al*. (2000) have shown a dominant role for the peroxisome proliferator activated receptor alpha (PPAR-α), and tumour necrosis factor alpha (TNF-α) in the process (Holden *et al*. 2000). For the peroxisome proliferating chemicals at least, a proportion of the pleiotropic response seen in the liver is specific to the rodent and species such as the guinea pig and human appear to be refractive to the majority of the adverse liver effects including the increases in cell replication and decreases in apoptosis seen with this class of chemical (James & Roberts 1995; Lake *et al*. 2000).

An additional complicating factor in this process is that the same chemical can simultaneously induce opposite effects in different organs at the same time. This has been demonstrated in rats given pregnenolone-16 alpha

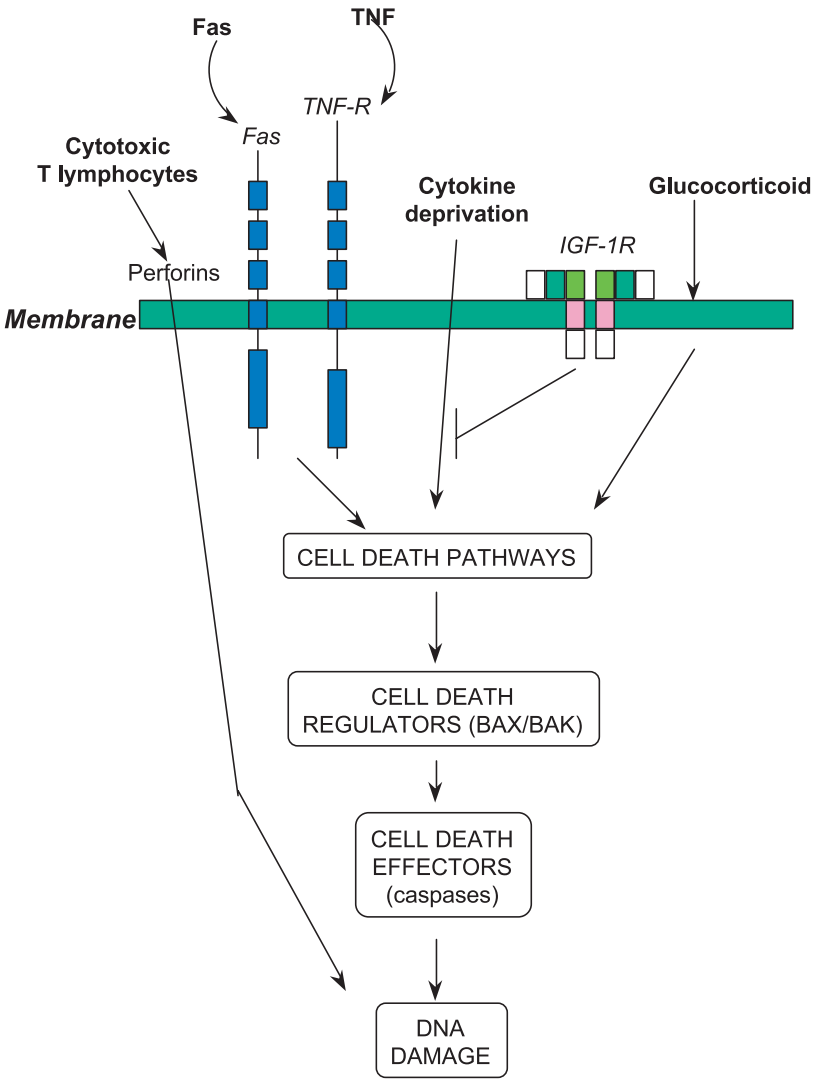

Figure 10. A model for the induction of apoptosis by a range of different stimuli including the interaction of certain cytokines with their respective receptors. Interaction of the Fas ligand or TNF with their respective receptors induces apoptosis as does administration of glucocorticoids and deprivation of cytokines from cell culture medium. Activation of the IGF-1R receptor is known to be able to block apoptosis in cells in culture (adapted from Meager 1998).

carbonitrile which decreases the levels of TGF-β1 protein in the liver, but increases the levels of the same peptide in the thyroid, while at the same time decreasing the levels of apoptosis in the liver and increasing the levels of apoptosis in the thyroid (Kolaja & Klaassen 1998). Once again the data advises caution in extrapolating the effects of a particular cytokine observed in one tissue to a second tissue, let alone from one species to a second.

Cytokines and chemically-induced allergy

Respiratory and dermal sensitization are important occupational hazards of the chemical, pharmaceutical and manufacturing industry (Bernstein 1996; Banks & Tarlo 2000). Allergy results from the exposure of susceptible individuals to chemicals which are able to activate the immune system, most probably through interaction with protein, and the subsequent perception by the immune system of the chemical/protein hapten as an immunogen. The most important cell involved in allergy is the T lymphocyte, and the control of migration, proliferation and differentiation of activated T lymphocytes is co-ordinated by cytokines of various types (Salluso *et al*. 1998). The activity of any single cell may be mediated by several different cytokines and their respective receptors in sequence in any one action.

The analysis of cytokine receptor mRNA levels has been advocated as a sensitive technique for predicting the immuno-modulatory potential of drugs and chemicals and Vandebriel *et al*. (1998) have applied this technology for monitoring the effects of the environmental contaminants, hexachlorobenzene, bis (tri-*n*-butyltin)oxide and benzo(a)pyrene, on splenic

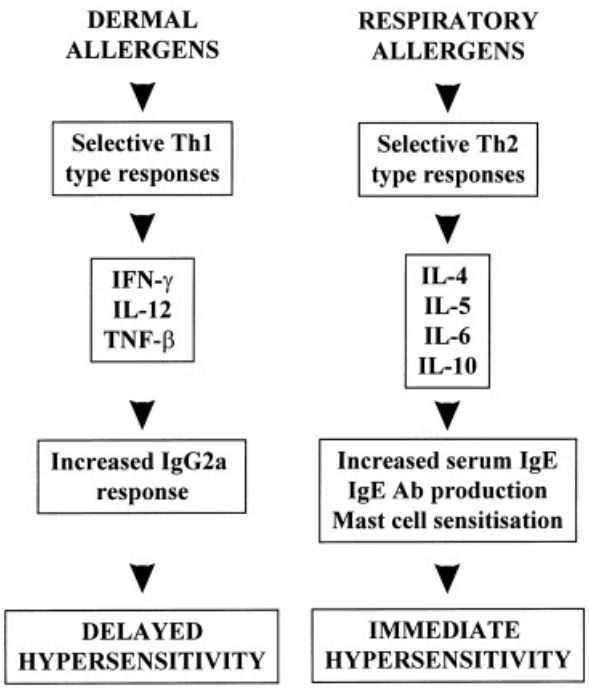

Figure 11. Cytokine fingerprinting in the distinction of respiratory from dermal sensitization. (adapted from Kimber & Dearman 1997).

lymphocytes. They showed both increases and decreases in IL2-Ra and IFN-γ mRNA levels dependent upon which chemical was administered while levels of IL-4 mRNA remained unaltered by treatment. The assays were reported to be among the most sensitive predictors for the immunotoxicity that these chemicals show in animals while also providing information on the mechanism by which the chemicals induce thymic atrophy.

It has been shown that the fundamental processes involved in respiratory and dermal allergy show some crucial differences that affect the subsequent cellular and cytokine responses to each allergy type (Van Loveren *et al*. 1996; Yokota *et al*. 1998). Investigative studies have shown that the T cells involved in the two processes are different and that they secrete different cytokines in response to chemicals that cause respiratory vs. dermal sensitization. Those T lymphocytes that are involved in dermal sensitization secrete type 1 cytokines produced by type 1 helper (Th1) cells, while those that are involved in respiratory sensitization have been shown to be type 2 helper cells (Th2) secreting type 2 cytokines. Type 1 cytokines include IL-2, interferon-γ (IFN-γ), IL-12 and tumour necrosis factor-β (TNF-β), while type 2 cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13 (Lipscomb & Wilder 1999). The differentiation of those chemicals and drugs that have the potential to induce contact sensitization from those that have the potential to induce respiratory sensitization has been achieved by phenotyping the cytokine response that each respective class of chemical elicits (Figure 11).

This theme has been adopted and developed to great effect by Dearman & Kimber (1999) to provide a qualitative method for assessing the potential allergenicity of chemicals under development, and also to differentiate those having respiratory sensitization potential from those that are dermal sensitisers (Kimber & Dearman 1997; Warbrick *et al*. 1998). The cytokine fingerprinting technique promises to have even greater application in the development of novel food sources such as those derived from genetically modified organisms where routine toxicological assessment procedures may be less than satisfactory (Basketter *et al*. 1994; Kimber *et al*. 2000).

Macrophages, cytokines and drug-induced tissue damage

Drugs and chemicals have been shown to cause tissue damage either directly, by metabolic activation to a cytotoxic product, or perhaps less commonly by activating resident tissue monocytes, neutrophils and lymphocytes or by recruiting them from the circulation (Brain 1992). The toxicity of hepatotoxic agents, such as acetaminophen and carbon tetrachloride, are significantly modified by the involvement of macrophages and the mediating chemicals that they secrete (Goldin *et al*. 1996; Orfila *et al*. 1999) Tissues such as the lung, liver and skin appear to be particularly susceptible and accumulations of macrophages are common responses to cytotoxic injury by any means in these tissues (Henson & Riches 1994). Subsequent release of reactive oxygen and nitrogen intermediates, cytokines and hydrolytic enzymes from these cells at the site of injury has the potential to exaggerate the injury process (Figure 12). Whereas some of these products, such as hydrogen peroxide and nitric oxide, can injure cells directly, other mediators such as collagenase and elastases can degrade the extra-cellular matrix, while cytokines such as colony stimulating factors, chemotactic factors, TNF-α and IL-6, promote inflammatory cell infiltration, cell proliferation and activation (Fujita *et al*. 1995).

The process of macrophage activation is tightly regulated by cytokines. However, as with many biological mediators, over-production of cytokines, such as IL-1 and TNF-α, or aberrant regulation of their release,

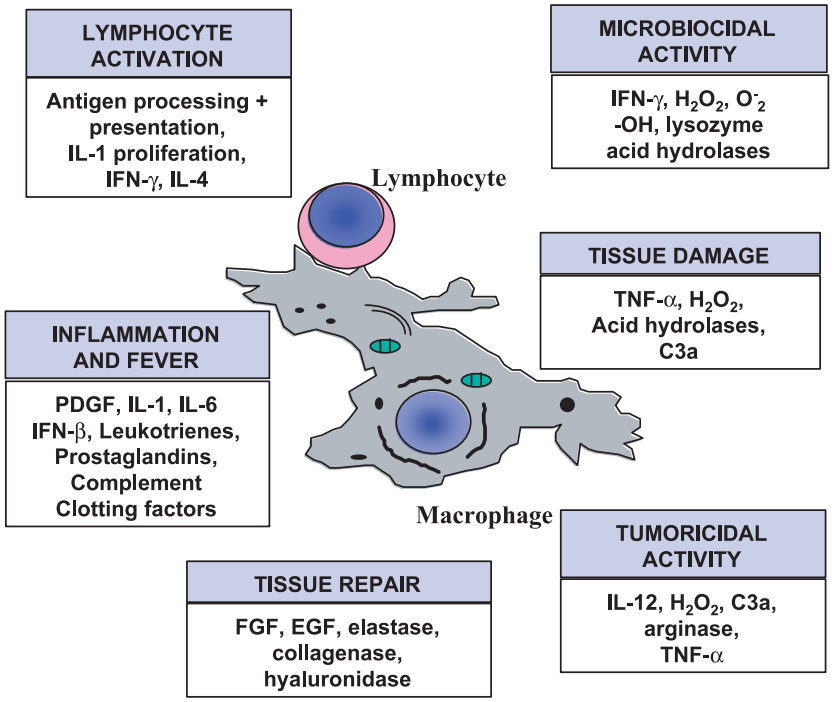

Figure 12. Putative interaction of macrophages with antigens in the initiation and mediation of chronic inflammation and tissue repair. The cell is central to the control of cytokine activity.

can lead to amplification of the inflammatory process resulting in increased tissue damage. Examples of this can be seen in ischemia re-perfusion injury, multiple organ failure and acute respiratory distress syndrome (Laskin & Pendino 1995).

Methodology for the study of cytokines

Any *in vivo* assay for cytokine production and release under experimental conditions will be fraught with difficulties based on the transient, and local (paracrine), function of the majority of cytokines. Hence plasma measures will have limited application unless the pathology under investigation is either very severe or is systemic (Whiteside 1994; Delarche & Chollet-Martin 1999). Organ or tissue-specific events, such as inflammatory conditions of joints and tendons, will largely preclude relevant measures of cytokines from plasma. In this respect the assays developed have largely reflected these limitations imposed upon systems (House 1999).

Bioassays

The original assays for cytokines depended on an assessment of the biological activity of a given cytokine as tested on a target cell in systems known as *bioassays* where a cytokine of unknown activity is assessed with reference to a known biologically active cytokine molecule (Mire-Sluis & Thorpe 1998). These assays establish dose–response relationships and many different biological systems are available (Thorpe *et al*. 1992). Such systems are cell proliferation assays using murine thymocytes (for IL-1), cytotoxicity tests (for TNF-α; TNF-β), antiviral activity, induction of MHC class II molecules (for IFN-γ), chemotactic activity (for IL-8) and inhibition of cytokine secretion (IFN-γ on IL-10 production). A major drawback of these types of bioassays is their lack of specificity due to the fact that several cytokines can exhibit similar or even synergistic activity. They also take time (24–96 h) and are reported to be poorly reproducible (Bienvenu *et al*. 1998).

Immunoassays (ELISA, RIA)

This methodology relies on the use of antibodies generated against each cytokine. In contrast to bioassays these methods of studying cytokines give fully quantitative data on changes in cytokine concentrations and show high specificity due to their use of monoclonal antibodies. They are able to distinguish cytokines having the same biological activity, e.g. IL-1a from IL-1b, and both radioactive and enzyme immunoassays exist. As with other methods that rely upon the use of antibodies, these assays detect the presence of apoprotein and are unable to prove activity in the cytokines that they detect

(Dinarello 1992; Whiteside 1994). Immunoassays generally are reported to be less sensitive than bioassays.

Solid phase enzyme-linked immunospot assay (ELISPOT)

This modification was introduced by Czerkinsky *et al*. (1988, 1991) for the assessment of cytokine production by activated cells *ex vivo*. The end product is an assessment of the colonies of cytokine producing cells which can be quantified by counting the number and area of coloured spots using image analytical methodology. The method is reported to be highly sensitive in detecting the presence of cytokines in individual cells present at low frequencies (Fujihashi *et al*. 1993; Karulin *et al*. 2000) and to be applicable for large scale screening applications.

Flow cytometry

Flow cytometry is a sensitive method for the quantitative analysis of changes in the cellular makeup of blood and tissues by the use of immuno-fluorescent markers to distinguish the different cellular subpopulations. The majority of the useful markers are antibodies and the technique has been used extensively in the study of changes in populations of lymphocytes in diseases of the blood (Parker 1988; Kipps *et al*. 1992). The methodology used for the study of cytokines by flow cytometry is essentially the same as that established for any other fluorescent marker used in the technique (Porter *et al*. 1996). Flow cytometry has been used to great effect to study cytokine changes in infectious, neoplastic and inflammatory disease in humans (Lucey *et al*. 1996) and techniques are now available for the study of both plasma membrane and intracellularly located cytokines (Jung *et al*. 1993). The ability to analyse the intracellular cytokine response under different conditions has greatly added to the value of investigations on cytokine control. A major advantage of flow cytometry is its ability to characterize not only the cytokines being produced but also the phenotype of the cells producing the cytokine simultaneously.

The major disadvantage of flow cytometry is its reliance upon single cell preparations and its inability to provide spatial information within intact organs and tissues. Since cytokine function is under tight regulatory control, the local environment, in the vicinity, for example, of inflammatory reactions, is likely to be considerably more relevant than systems at a distance from the affected areas, e.g. in the plasma or circulating lymphocytes.

Immunocytochemistry (cytokine localization in cells and on the cell membrane)

A discussion of the immunolocalization of cytokines in cells (immunocytochemistry) has been deliberately separated from that in tissues (immunohistochemistry), on the grounds that some fundamental differences exist in sample preparation such that the difficulties associated with the use of the two techniques are significant.

This methodology depends upon the availability of monoclonal and polyclonal antibodies, specific for each cytokine, together with preparation methodology appropriate for retaining the antigenicity and allowing access of the antibody to the cytokine protein. As a result a large amount of data exists for the study of cytokines in lymphocytes and monocytes from the circulation or grown from tumour explants where cell separation and preservation is relatively simple (Dobbeling *et al*. 1998; Yamada *et al*. 1998; Hjorth-Hansen *et al*. 1999). The preparation of cellular imprints from lymph nodes is also a relatively simple procedure that has been used for many years in the diagnosis and differentiation of various lympho-proliferative disorders (Torlakovic *et al*. 1999; Lonn *et al*. 1995) and their adaptation for cytokine immunocytochemistry is relatively simple. These preparations have been used for immunocytochemistry of a variety of different protein markers in various types of cancer and standard protocols have been developed for the techniques (Giorno 1983).

A modification of the imprint methodology involves the mechanical dissociation of the component cells of solid tissue, for example, by passing the tissue through a synthetic mesh membrane (Szucs *et al*. 1994). The dissociated cells are then in a similar form to those present in blood and their subsequent immobilization onto glass slides and immunostaining can be readily achieved using methodology similar to that used in flow cytometry (see Figure 13). Experiments using cells prepared in this way have recorded changes in the expression of the receptors to IL-4, IL-6 and IL-2 in human melanoma, ovary and breast carcinoma (Chen *et al*. 1991; Rimoldi *et al*. 1993; Obiri *et al*. 1994). The technique of mechanical dissociation seems to provide an ideal compromise between maintaining morphological preservation without unduly compromising the antigenic properties of the proteins which are to be studied by immunolocalization. The proteins with cytokine activity, in particular, seem to be present in low amounts *in vivo*, to be transiently expressed and to be especially susceptible to fixation denaturation. All these factors conspire to make the study of cytokines by *routine* fixation and preparation procedures generally

unsuitable. Suitable fixation procedures utilize a paraformaldehyde/saponin preparation (Sander *et al*. 1991) which allows appropriate fixation and permeabilization of the cell membranes to allow antibody access without excessive denaturation of the antigenic determinants on the cytokine molecules.

Immunohistochemistry (cytokine localization in tissue sections)

In contrast to the relatively easy preparative techniques used for flow cytometry and immunocytochemistry of cytokines, their study in tissue sections carries a number of additional problems in terms of preservation of tissue morphology and cytokine antigenicity. It has been found that in the use of freshly *sectioned* frozen tissue, immobilization of soluble proteins is essential in preventing their loss and redistribution during the subsequent immunohistochemical staining protocol (Thorpe *et al*. 1992). Unfixed cryostat sections have optimal antigenic preservation but frequently show poor localization of cytokines due to a combination of inadequate morphological preservation, low constitutive expression of the cytokine and, most importantly and frequently underestimated, leaching of the target protein from the sectioned cells.

In terms of immunohistochemistry the 'inflammatory cytokines', such as the interleukins, can be separated from the more commonly referred to 'growth factors' in terms of their ease of localization. The former carry particular difficulties in terms of their preservation, and standard techniques have had to be modified to accommodate the fact that they are generally expressed at low levels and are particularly susceptible to fixation denaturation. Standard fixation protocols almost invariably denature both cytoplasmic and membrane-bound cytokines unless very brief protocols are employed (Hazen-Martin & Simson 1984; Anttila *et al*. 1992) which may additionally lead to antigen loss as in unfixed material. In order to counter this Schrijver *et al*. (2000) have employed a pararosaniline based fixation schedule which is reported to provide good morphological and antigenic preservation of cytokines in human and mouse spleen. It remains to be seen whether or not this technique is adopted by other laboratories but if successful it will be a powerful ally in this aspect of cytokine study in an area which has yet to fulfil its potential in terms of contributing to our knowledge of cytokines and how they work.

In contrast to the situation with the inflammatory cytokines, immunohistochemical studies on the growth factors have shown them to be considerably more tolerant of histological preparative procedures such that considerable success has been achieved with even relatively routinely processed material (Yabu *et al*. 1993; el-Sherif *et al*. 2000). The major difference is almost certainly related to differences in expression levels generally between the inflammatory cytokines and the growth factors which frequently show high levels in tissues undergoing growth stimuli.

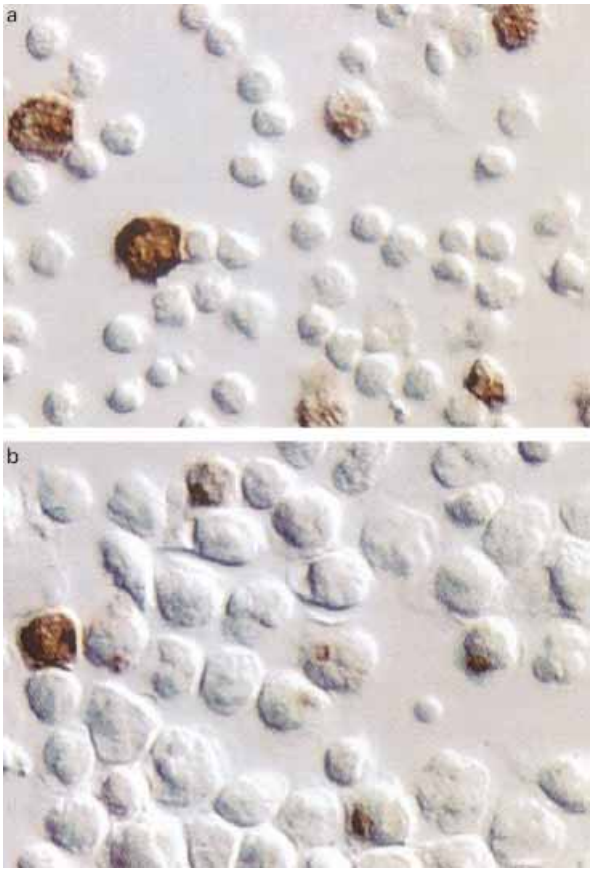

Figure 13. Immunocytochemistry of cytokines in human peripheral blood mononuclear cells following overnight exposure to lipopolysaccharide *in vitro* in the presence of the protein transport inhibitor, monensin. (a) Intracellular localization of IL-6. (b) Intracellular localization of IL-5. Nomarski optics, 400× magnification. Reprinted with the permission of Dr M. Garcia-Valcarcel, BD Biosciences.

In situ hybridization (mRNA expression in sections and cells)

This methodology detects the mRNA for cytokines rather than the protein itself. The method is complementary to immunohistochemistry/immunocytochemistry and can be used on both separated cells and on tissue sections. It has similar problems associated with it

to that seen with the immunochemical techniques but in general mRNA appears to be more resistant to fixation denaturation than the corresponding protein and hence may have more widespread application than the methods identifying cytokine proteins themselves (Suliman *et al*. 1999; Slominski *et al*. 1999).

Toxicogenomics

The advent of large scale genome sequencing and the technological application of these genes to so-called DNA chips or microarrays allows the simultaneous analysis of many thousands of genes from tissues and cells obtained from diseased or drug-treated animals and cells including those from humans (Pennie *et al*. 2000). The methodology involves the extraction of mRNA from cells or from tissues which is subsequently labelled and hybridized to the microarray and visualized using a phosphorimager. The results are analysed using image analysis software which can compare control with test samples (Brown & Botstein 1999) and provide quantitative information on the degree of induction or down-regulation of any particular gene such as a cytokine. The commercial availability of the microarrays allows the serial analysis of gene expression for thousands of genes simultaneously in a single experiment (Derisi & Iyer 1999; Hiltunen *et al*. 1999; Pollack *et al*. 1999; Emilien *et al*. 2000; Lockhart & Winzeler 2000; Rockett & Dix 2000). In this way it should ultimately be possible to look for changes in cytokine expression across a much broader range than with the techniques currently available and avoid having to decide beforehand which cytokine to study.

Proteomics

An adaptation of protein gel electrophoresis, for which the term 'proteomics' has been coined, allows the simultaneous analysis of many hundreds of proteins in the technology known as 2D polyacrylamide gel electrophoresis (Anderson *et al*. 2000). In combination with mass spectrometry it has been used to study the altered protein expression following administration of EGF, TGF-α or the peroxisome proliferator, nafenopin, to hepatocytes *in vitro* (Chevalier *et al*. 2000; Davison and Roberts 2000), and the effects of the peroxisome proliferator, Wy 14,643, on the livers of obese mice (Edvardsson *et al*. 1999). The technology can be time consuming with the analysis difficult and relatively insensitive. A development of this has been recently attempted whereby so-called 'protein-chip arrays', comparable to those produced for DNA, have been made (von Eggerling *et al*. 2000). The technology is clearly still in its infancy but, like the genomics area, also has the potential to allow the study of the expression of large numbers of different proteins, including cytokines, simultaneously (Godovac-Zimmerman *et al*. 1999; Soskic *et al*. 1999; Steiner & Anderson 2000) with all the advantages that this brings.

Cytokines in therapy

Safety evaluation of novel cytokine therapies requires detailed animal toxicity studies with an increasing need for more specific tests for detecting changes in immune status, such as immune deficiency, auto-immunity or hypersensitivity to the drug (Panitch *et al*. 1987; Arnason & Reder 1994), or increased or decreased inflammatory reactions at tissues sites unrelated to those at which the cytokine would be predicted to act (Car *et al*. 1999; Lang *et al*. 1987; Gearing *et al*. 1989). Neurotoxicity, in the form of demyelination, has also been observed in humans in the therapeutic use of TNF-α (Sidhu & Bollon 1993; Hieber & Heim 1994) and increased confidence is needed that effects such as these would not be missed in preclinical animal studies. Current batteries of animal tests have proven to be insensitive in the detection of these endpoints (Basketter *et al*. 1994) but refinements in the testing protocols have significantly improved their discriminative ability in predicting these effects (Institoris *et al*. 1998; Hinton 2000; Kuper *et al*. 2000). Animals, transgenically altered in their cytokine expression to study basic biological properties of cytokines (Ryffel 1997), have also shown disease states, such as arthritis and other chronic inflammatory diseases, which are rare in nongenetically altered mice strains (Keffer *et al*. 1991; Allison *et al*. 1992). It is also highly probable that transgenically altered animals will be used in the future to provide enhanced animal models for the prediction of certain types of immune response for which unaltered animals are insensitive (Barton *et al*. 2000; Bugelski *et al*. 2000; Nakao *et al*. 2000). The use of these animals will confront toxicology with a new set of challenges which will require a flexible approach in understanding the events observed. It is a challenge that the science will increasingly have to grappling with.

Limitations in the study of cytokines in toxicology

During the last 15 years a body of literature has been published describing various assays for investigating changes in cytokines in fluids and tissues. The very fact that cytokines act on numerous target cells make it difficult to define a precise role for any given molecule.

They act at very low concentrations (10^{-10}–10^{-15}M) and their circulating concentrations can be even lower, while their predominantly paracrine function means that the highest concentrations are seen at their site of action. They also show extremely short half lives leading to brief peaks which require precise timing to detect. Certain cytokines, such as IL-6, have been shown to exhibit circadian rhythms in secretion such that the highest plasma concentrations are seen in the morning (Arvidson *et al*. 1994; Redwine *et al*. 2000). In terms of their response to chemical administration they show temporal induction where concentrations may peak in a matter of minutes, or for those involved in cellular transformation their induction may take many months. Any particular cellular change will often result in the induction of multiple cytokines and their effects are often multifactorial and pleiotropic and dependent upon the tissue changes present at the time of their release. In addition, of particular concern in predictive studies where early events are used to predict the longer term outcome of exposure, induction of the same cytokine may have different and multiple outcomes. Selection of which particular cytokine to measure is critical and in an undefined system the choice may present difficulties. Similarly, although plasma cytokine measurements provide the most convenient way of monitoring, they may not be specific or sensitive when effects are occurring locally in a tissue such as that occurring with some inflammatory joint diseases where assays at the point of tissue changes may provide considerably more specificity and sensitivity of the response.

Despite all of these caveats there is intense interest in the pharmaceutical industry to discover the basic factors involved in the cytokine control of disease processes in general with a view to modulating their activity by the use of cytokines themselves, by the use of synthetic molecules that antagonize or agonize the action of the cytokine proteins at the site of action, i.e. their receptors, and by the use of antisense oligonucleotide technology where the production of the mRNA for the respective cytokine is inhibited (Armendariz-Borunda *et al*. 1997; Dachs *et al*. 1997; Ojwang & Rando 1999).

An increasing understanding of how cytokines modulate cell growth and differentiation, cell migration and activation in response to chemicals, and under both normal and altered physiological states promises to yield enormous benefits in medicine and biology, including toxicology. A critical aspect to the success of this venture will be the accurate and sensitive evaluation of changes in cytokine concentrations, particularly at the site of action. Only then can a realistic expectation be made of fathoming the complex relationships that occur between tissues and drugs, and subsequently between cytokines and the tissue responses that modulate the therapies to which drugs are applied (Borden *et al*. 1995; Dawson & Wynford-Thomas 1995).

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